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(54) Detection of enterotoxigenic E. coli

(57) An method of detecting heat labile toxigenic strains of E.coli in a sample comprises filtering the sample through a membrane filter to trap cells of any such strains present, incubating the resulting used membrane filter with a selective medium which contains at least approximately 0.1% by weight a surface active agent selective for Gram-negative microorganisms and which also contains lactose in a concentration no greater than 5 g/litre and is permissive of heat labile toxin-production by E.coli, placing the said membrane on a medium containing antitoxin to E.coli heat labile toxin and incubating the said membrane and antitoxin-containing medium to produce zones of precipitation corresponding to any colonies of heat labile toxigenic E.coli organisms on the said membrane. The invention also encompasses selective media and antitoxin-containing media and a kit which includes a filter having a pore size of 0.1 μm to 0.65 μm.

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SPECIFICATION

Detection of enterotoxigenic e coli

This invention relates to a new method which is designed to simplify the detection of heat labile toxigenic strains of Escherichia coli with a view to replacing current standard tissue culture methods. The method permits the study of toxigenic E. coli in a wide range of source materials and it is therefore not limited to use in the identification of enterotoxigenic E. coli from clinical sources alone. Furthermore, the invention is also applicable to cholera and other enterobacteria which produce a cholera-like toxin. The invention also relates to media which are especially adapted for use in the method of the present invention and to a kit.

As little as 10 years ago the aetiology of diarrhoea could only be determined in about 20% of cases. In 1945 it was first established that E. coli could cause severe diarrhoea and frequency result in death particularly in infants. Since then intensive research has demonstrated that E. coli may be the single most important cause of diarrhoea in some developing countries (I.C.D.D.R., B Annual Report, 1981). Furthermore this species is now divided into 3 groups of which two are clearly distinguishable on the basis of pathogenesis and clinical criteria as follows:–

1: Enterotoxic E. coli (ETEC)

Two types of enterotoxin are produced by members of this group. This is one important cause of watery diarrhoea in infants, young children and adults in developing countries. It is also one of the principal causes of travellers' diarrhoea in visitors to ETEC-endemic areas and is clinically indistinguishable from mild cholera.

2: Enteroinvasive E. coli (ETEC)

Cases of diarrhoea caused by this group appear to be fewer in number than the former group. They are invasive in the gut and have a pathogenesis similar to shigellosis.

3: Enteropathogenic E. coli (EPEC)

The term EPEC is clearly applicable to both groups 1 and 2, but historically the term was first applied to E. coli isolated from nursery outbreaks of infantile diarrhoea. These early isolates belonged to specific serotypic groups which were identified before the mechanisms of pathogenesis had been elucidated. This term is now used with less confidence by the majority of diagnostic laboratories, which have the facilities for confirming pathogenic potential (bioactivity). The commercial serotyping schemes are now inadequate and invalid as a method for establishing the pathogenicity of presumptive pathogenic isolates as the O and K antigens are not necessarily associated with plasmid-mediated pathogenic factors. This is particularly true in developing countries where an increasing number of isolates from clinical cases do not belong to the classical European serotypes and are untypable.

In 1968, studies in Calcutta showed that strains of E. coli could be isolated from adult patients who had cholera-like illness, but from whom no Vibrio cholerae could be isolated (Corbach, S.L. et al, 1971; Sack, R.B. et al, 1971). These did not belong to conventional EPEC serotypes, but were found to produce enterotoxins. The clinical picture upon admission was indistinguishable from that of cholera, including production of 'rice water' stool, profound dehydration and shock. The only clear difference was relative brevity of the ETEC diarrhoea; stool output ceased within 24–30 hours of admission without antibiotic therapy. Incubation studies showed that during the acute phase (i.e. 16–24 hours), the gut population of E. coli was 10^7–10^9/ml; in the proximal small intestine (consisting of only one or two serotypes). These disappeared during the convalescent phase. The physiological abnormalities in fluid and electrolyte movement, in the intestine were similar in kind, although less in magnitude to those seen in cholera (Banwell, J.G., 1971).

Using cultural techniques similar to those used in the production of cholera toxin, it was possible to demonstrate the production of two types of enterotoxins by E. coli.

1: Heat Stable (ST) Toxin

A low molecular weight polypeptide (1,500–5,000) toxin which withstands temperatures of 100°C for 15 minutes. Culturally, it is produced by vigorous aeration during incubation (Sack, R.B., 1975). It is responsible for the stimulation of cyclic AMP (Sack, R.B., 1980).

2: Heat Labile (LT) Toxin

A high molecular weight polypeptide (73,000) toxin, which is destroyed by temperatures of 60°C for 30 minutes. It is non-dialysable and immunogenic. Culturally, it is produced by still cultures, during incubation, with a large surface-area to volume ratio. The toxin shows marked increase in anti-E. coli enterotoxin titre, in paired sera of patients (Sack, R.B., et al. 1974; Guerrant, R.L., et al. 1974; and Evans, D.G., et al. 1973). It is responsible for the stimulation

of cyclic AMP (Evans, D.J., et al. 1972; Hewlett, E.L., et al. 1974 and Kantor, H.S., et al. 1974).

On the basis of the types of toxins produced ETEC strains can be classified as:–

1: LT Strains
2: ST Strains
3: LT/ST Strains

In some studies, all human strains show both LT and ST toxins (Dupont, H.L., et al. 1976; Evans, D.G., et al. 1975) i.e. LT/ST strains. In other studies either only LT strains or only ST strains were found (Guerrant, R.L., et al. 1975; Merson, M.H., et al. 1976).

Studies on Travellers' diarrhoea in Mexico (Gorbach, S.L., et al. 1975) indicate that the LT-producing strains were responsible for diarrhoea. Other similar studies indicated a higher proportion of LT strains or LT/ST strains than just ST strains (Morris, G.K., et al. 1976; Echeverria, P., et al. 1981).

The picture varies in different geographical regions for diarrhoea (infantile or travellers'). For example, in Mexico and Morocco LT/ST strains predominate. In Kenya, LT strains are most common whereas in Bangladesh ST strains seem to occur slightly more commonly than LT/ST strains (Bulletin, W.H.O., 1980). Preliminary work carried out on Malaysian isolates of infantile and childhood diarrhoea suggests a larger proportion of LT than LT/ST or ST (Vadivelu, J., 1982).

The above information suggests the importance of LT in Travellers' diarrhoea and it also occurs singly or in combination with ST in most infantile and childhood diarrhoea.

Watery diarrhoea, including ETEC, are successfully and primarily treated by rehydration therapy. The W.H.O. Diarrhoeal Disease Control Programme is promoting this procedure globally. However, rehydration therapy is no substitute for prevention and does nothing to prevent reinfection in insanitary environments. Prevention of diarrhoeal disease depends upon sustained public health interventions but funds for this work are very limited in developing countries. Cost effective public health strategies may be developed when the major transmission route of the dominant aetiological agents have been identified. There is therefore an urgent need for simple, economical and rapid methods for tracing and quantifying toxigenic E. coli populations in environmental and clinical samples in developing countries.

There are basically three types of test which have been used so far for identification of LT-toxin. Thus, a first group of tests are based upon in vivo experimentation using live animals. These tests depend upon the phenomenon of fluid accumulation, due to disordered fluid and electrolyte movement in the gut, when a toxin is present, and require the initial stage of preparation of crude toxin preparation from the sample being tested. This crude toxin is then inoculated into the gut of the test animal and the presence of an LT toxin indicated by fluid accumulation. Although these tests are very sensitive they are costly and time consuming and very experienced laboratory staff are required in addition to animal unit facilities. A second group of tests involve tissue cultures and these tests are very widely used. Although they are more economical than animal tests, a crude toxin preparation as referred to in connection with the animal tests is still required and experienced laboratory staff and tissue culture facilities are an absolute essential. The presence or absence of toxin is indicated by the cytopathic effect, if any, on the cells of the crude toxin preparation. Both animal and tissue culture tests are usually used in conjunction with "colony pooling" (Merson, et al. 1979). The pools usually consist of 10 colonies from a primary isolation plate. Less colonies may be used but obviously with a lower probability of detecting toxigenic-strains. Colony pooling does not give quantitative data as to how many of the ten colonies are toxigenic or qualitative data as to which of the 10 colonies are toxigenic. It is possible that the normal gut flora may contain small numbers of potentially toxigenic strains but is unlikely that a false identification of the aetiology of an infection may result from this, although the carrier rate of ETEC in the normal population cannot be readily studied with currently available methods. It is probable however that a substantial number of ETEC infections remain undiagnosed due to the insensitivity even of colony pooling.

The third group of tests make use of the immunogenic property of E. coli LT toxin. These tests involve an antigen-antibody reaction and thus require an antitoxin which is an antibody against LT- or cholera toxin. However, there is an urgent need for a test which permits routine assay for toxin production and which would enable identification and quantification of LT-toxigenic isolates. Furthermore, all existing tests require at least three and some as much as seven days before results are obtained. Furthermore, prior tests often involve sub-culturing which may induce plasmid loss (Evans, D.J., et al. 1977). Random picking of colonies also carries the risk of toxigenic colonies being missed. The present invention aims to avoid those difficulties.

Accordingly, the present invention provides a method of detecting heat labile toxigenic strains of E. coli in a sample, which method comprises filtering the sample, if necessary after dilution, through a membrane filter having a pore size small enough to trap cells of any such strains

present in the sample and made of a material sufficiently free from extracellular protein-binding capability, and incubating the resulting used membrane filter with a selective medium which contains in an amount of at least about 0.1% by weight a surface active agent selective for Gram-negative microorganisms and which also contains lactose in a concentration no greater than 5 g/litre and is permissive of heat labile toxin-production by E. coli, if the thus-incubated membrane carries lactose-fermenting colonies placing the said membrane on a medium containing antitoxin to E. coli heat labile toxin and in which a complex of the said toxin and antitoxin is insoluble and incubating the said membrane and antitoxin-containing medium to permit toxin migration into the antitoxin-containing medium and the production of zones of precipitation corresponding to any colonies of heat labile toxigenic E. coli organisms on the said membrane. The same method may be applied to cholera and other enterobacteria which product cholera-like toxins and the invention includes this.

In general, the above method may be employed with any particular sample. In other words, as an integral part of the procedure a raw microbial population is isolated from any source, e.g. environmental (water), food or clinical sources. Of course, if the sample is primarily solid it must be diluted prior to filtration using conventional techniques. The development of a primary selective isolation medium which enables a differential colony count of E. coli to be produced and which also does not inhibit toxin production unduly, the development of an antitoxin-containing medium adapted to permit the detection of toxin/antitoxin complex as a precipitate and the use of a membrane filter are, in combination, essential to the present invention.

Membranes may be of a cellulose acetate, but *not* nitrocellulose (cellulose nitrate) because they bind a proportion of the protein released. There are advantages in using hydrophobic edged membranes as this reduces the risk of under-membrane contamination during and after transfer to the antitoxin-containing medium—but this is outweighed by the currently higher cost of the membranes—and a careful worker should readily prevent such contamination by routine aseptic techniques.

In the present method commercially available bacteriological membranes may be used. The pore size may be as small as about 0.1 μm (below which size the operation is usually impractical) or as large as 0.65 μm. Sizes of 0.7 μm and above are preferably avoided, and an ideal range is 0.22 μm to 0.45 μm. e.g. 0.45 μm.

The key diagnostic reagent necessary for the immuno-assay is an antitoxin. The E. coli, LT Toxin and the V. cholerae enterotoxin are functionally, structurally and immunogenically similar (Sack, R.B., 1980). The E. coli toxin has been found to cross-react with the V. cholerae antitoxin (Gyles and Barnum, 1969). However, only the cholera toxin (Sigma) is available commercially; this is therefore the preferred choice of antigen rather than crude LT toxin. The commercial product is purer and therefore a more specific form of antiserum can be obtained. With regard to producing antiserum for use in an immuno-assay, it should be borne in mind that a high titre and avid antiserum is required. Therefore, a proven immunisation protocol should be used such as the following:–

An aqueous solution of cholera toxin at 100 μg/ml in phosphate buffered saline is first emulsified with non-ulcerative Freund's incomplete adjuvant (Marcol) in the ratio of 1 volume of toxin to 1.5–2.0 volumes of the adjuvant. The mixture is emulsified until stable, the water being introduced into the oil first. The emulsion is then injected intradermally in 50 μl volumes into the shaved back of 2 female white half-lop rabbits.

The Elisa technique may be used to detect and monitor the serum level of antitoxin produced in rabbits thus-inoculated (Enzyme Linked Immunoabsorbent Assay—Sack, D., et al, 1980).

Present commercially available selective isolation media are unsuitable for use in the method of the present invention since although they permit selection of E. coli they are generally unsuitable for toxin production. This may be due to the acid pH formed in such media, low pH being inhibitory for toxin production (Kunkel, S.L., et al. 1979). However, it is also possible that other constituents of commercial media may inhibit toxin production. Accordingly, it was necessary to develop an entirely new medium for the present method which enabled selection for E. coli and yet was permissive of toxin production. Work has shown that a range of surface active reagents are non-inhibitory to toxin production. In addition, in the absence of fermentable carbohydrate in the medium, the pH increased from 7 to above 8 when incubating toxigenic cultures of E. coli. Faecal coliform isolation media, however, routinely incorporate lactose to differentiate acid-producing colonies at the incubation temperature of 44°C. However, experiments showed that whilst lactose concentrations of up to 5 g/litre permit toxin production, higher concentrations completely inhibit detectable toxin production. This is an undexpected and highly important result since in commercial faecal coliform media lactose concentration varies from 10 to 30 g/litre. It could be argued that lactose fermentation induces and acid pH which in turn inhibits toxin production. However, experiments have shown that even raising the initial medium pH to 8.5 such that the likelihood of a subsequent reduction of pH during fermentation to below 7 is minimised does not alter the fact of inhibition of toxin production with lactose concentrations of above 5 g/litre.

It is absolutely critical in the present invention that the selective medium has a lactose concentration which does not excedd 5 g/litre and also that the surface active agent selective for Gram-negative microorganisms should be present in the medium in an amount of at least about 0.1% by weight. In the case of the latter, concentrations of, say, 0.08% may be used but 0.1% is a reasonable experimental minimum. Concentrations appreciably below 0.1% do not work properly.

Preferred selective agents for the selective medium are Tergitol (BDH), sodium lauryl sulphate, bile salts (bile salts No. 3 from Oxoid are appropriate), or Teepol (BDH610). The amount of Tergitol used is preferably 0.1% by volume, the amount of sodium lauryl sulphate preferably 0.1% by weight, the amount of bile salts preferably 0.1% by weight, and if Teepol is used, the amount is preferably 0.4% by volume.

It is also highly preferred that the selected medium contains an indicator and Phenol Red or Cresol Red can be used for this purpose. Cresol Red is the most preferred indicator and is used in an amount of 0.2 g/litre.

Insofar as the other ingredients of the selective medium are concerned, Tryptone soy broth or Trypticase soy broth may be used in an amount of 30 g ± 1 g/litre of medium. Yeast extract may also be included in the medium, e.g. in an amount of 12 g/litre.

The antitoxin-containing medium of the present invention usually contains an antiserum derived from an inoculation procedure using cholera toxin as described above. The highly preferred form of the medium is an antitoxin agar containing such antiserum, purified agar and either Tryptone soy broth or Trypticase soy broth. It is preferred that the Tryptone soy broth or Trypticase soy broth be present, per 1000 ml of medium, in an amount of 30 g ± 1 g, the purified agar present in an amount of 1.5% ± 0.2% by weight and the antiserum present in an amount of 0.5% ± 0.1 by volume. As with the selective medium, yeast extract can be present, e.g. in an amount of 12 g/litre.

Accordingly, below are given the compositions of preferred selective media and preferred antitoxin-containing media:-

SELECTIVE MEDIA (1000 ml)

1.	Tryptone soy broth or Trypticase soy broth	30 g ± 1 g
2.	Selective Agents:	
	Tergitol (BDH) or	0.1% v/v
	Na Lauryl sulphate or	0.1% w/v
	Bile salts No. 3 (Oxoid) or	0.1% w/v
	Teepol (BDH610)	4 ml l^{-1}
3.	Lactose	4 to 5 g/l
4.	Cresol Red	0.2 g/l
5.	Yeast Extract	12 g
6.	Distilled Water	Balance

pH adjusted to 8.5 prior to autoclaving

ANTITOXIN-CONTAINING MEDIA

1.	Tryptone soy broth or Trypticase soy broth	30 g ± 1 g
2.	Purified Agar (Oxoid L28)	1.5% ± 0.2% w/v
3.	Antiserum (used crude) Prepared by intradermal inoculation of 100 µl of cholera toxin in phosphate buffered saline mixed in 2 ml of non-ulcerative Freuds adjuvant (Marcol).	0.5% ± 0.1% v/v
4.	Yeast Extract	12 g
5.	Phosphate Buffer (0.3M)	1000 ml

In a typical procedure in accordance with the present invention, the sample for testing is first mixed with aqueous diluent if necessary (obviously not appropriate in the case of a water sample) and then filtered through a cellulose acetate membrane filter having a pore size of 0.45 µm. The membrane filter is then incubated for from 12 to 36 hours, e.g. about 18 hours, with a selective medium as noted above. The membrane filter is then examined, and, if it carries fermenting colonies (which are by definition, having regard to the conditions of incubation, able to ferment lactose) having faecal coliform morphology, it is placed on the surface of an agar plate loaded with an antitoxin-containing agar medium as described above and the combination of membrane and antitoxin-containing medium is then incubated for from 18 to 48 hours, e.g. about 24 hours. Both the primary incubation with the selective medium and incubation with the antitoxin-medium are preferably conducted at 44°C. Any toxin present migrates through the membrane during the course of the second incubation to produce circular zones of toxin-antitoxin precipitation concomitant with the overlying colonies on the membrane. In this way, toxigenic colonies may be identified and the proportion of toxigenic isolates in the population examined may be enumerated. The antitoxin-containing medium may be modified to enhance this precipitation by the incorporation of polymixin B or lincomycin, e.g. at a concentration of 2000 IU/ml and 90 µg/ml, respectively. Toxigenic colonies thus noted may, of course, be isolated and subjected to reference laboratory confirmation. It can be expected that toxigenic strains present will produce zones of precipitation in an antitoxin agar using this technique within 48 hours. Physical separation of toxigenic organisms on the membrane filter permits the diffusion of toxin through the membrane into the underlying antitoxin agar and, accordingly, an elegant analysis system is developed.

Thus, examples of the application of the present technique are as follows:-

1. *Human case of water diarrhoea*
   a) Dilute 1 ml or 1 g of faecal specimens 10,000, 100,000 and 1000,000 in Ringers.
   b) Filter specimen through bacteriological membrane filter.
   c) Incubate membrane on selective medium (as described at 44°C for 12–20 hr) and select membranes carrying 1–100 colonies for d).
   d) Transfer membrane to antitoxin agar and continue incubation for 24–48 hrs at 44°C.
   e) Examine for zones of precipitation in agar and retain those colonies which produced pp^{in} for further investigation.
   f) Report toxin producing isolates.

2. *Suspected pathogen contaminated water samples and beverages*
   a) Membrane filter triplicate sample and proceed as in 1b) to 1f).

3. *Suspected pathogen contaminated food*
   a) Homogenise 10 g of sample, prepare decimal dilutions to 10^{-6}.
   b) Filter specimens that are sufficiently diluted.

c) Proceed as in 1c) to 1f).

To evaluate the toxin-detection rate of the method of the present invention, the method was compared with the cytopathic effect on Vero cell lines using a reference collection of 20 E. coli supplied by Rowe (PHLS, Colindale, U.K.) The following is the Vero cell technique used.

Vero Cell LT-toxin reference assay (Kashiwazaki, M., et al. 1980):

For the assay the cells were stripped by Versene/Trypsin and suspended in 10 ml of maintenance medium. 0.5 ml of the cell suspension was added to 4.5 ml of fresh maintenance medium to obtain a total cell count, using a haemocytometer. Then, the appropriate amount of cell suspension was added to 19 ml of test medium to obtain a cell concentration of 10^5 cells/ml.

The test medium was made up as follows:–

To 90 ml of sterile distilled water add,

Cell medium 199	10.0 ml
Sodium Bicarbonate (7.5%)	0.6 ml
Fetal Calf Serum	1.0 ml
Penicillin/Streptomycin	1.0 ml
Cindomycin	0.1 ml
Hepes Buffer (1M)	2.5 ml

To each well on a microtitre plate 25 μl of test medium was added. To this 25 μl of test sample was added to the top row. Doubling dilutions of the test sample were then carried out until the penultimate row, at which point 25 μl was discarded. The last row therefore acted as a negative control. To each of these wells 100 μl of cells in test medium was added. The plate was sealed with a plastic sealant to avoid gas diffusion. It was then incubated at 37°C overnight.

The following day the bathing medium was discarded and the cells fixed with 70% ethanol for 10 minutes. The ethanol was discarded. The cells were then stained with Geimsa 1/50 concentration and left for 20 minutes. The stain was then discarded and the plates allowed to dry before the results were read using an inverted microscope.

Cytopathic effect was observed by cell membranes ending in tendrillar processes.

In this comparison, in the case of the present method three colonies of each isolate from the aforesaid reference collection of E. coli were grown on individual membranes and examined for zones of precipitation at 24 and 48 hours. The agreement between the 24 and 48 hour results was 90%, the discrepancy was due to the development of precipitation zones in two cultures during the second day of incubation. The discrepancy between the method at 48 hours and the Vero cell results was attributable to two extra positives being detected. Whether or not these were false positives is not clear but such results are still very encouraging. The following table gives the results of this comparison.

Comparison of LT-toxin detection using the Vero cell line and membrane filtration plus immunoprecipitation at 24 and 48 hrs.

E.coli Isolate No.	Vero cells	3 Colonies on each membrane filter (MF) isolation broth for 18-24 h then					
		24h antitoxin agar			48h antitoxin agar		
1	+	-	-	-	+	+	+
2	-	-	(+)		(+)	(+)	(+)
3	-	+	+	+	+	+	+
4	-	+	+	+	+	+	+
5	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+
7	-	-	-	-	-	-	-
8	+	+	+	+	+	+	+
9	+	-	-	-	+	+	+
10	+	+	+	+	+	+	+
11	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-
14	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+
18	+	+	+	+	+	+	+
19	-	-	-	-	-	-	-
20	+	+	+	+	+	+	+
Total Strong Positive	12		12			14	
Agreement		75%		90%			
			85%				

+ = Toxin detected: (+) + weak: - = no toxin detected

In addition to the above detection/analysis method and the novel media, the present invention also provides a kit for use in the direction of heat labile toxigenic strains of E. coli comprising a membrane filter with a pore size of 0.1 to 0.65 μm, e.g. 0.45 μm, a selective medium as defined above in accordance with the invention and/or an antitoxin-containing medium as defined in accordance with the invention.

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CLAIMS

1. A method of detecting heat labile toxigenic sterains of E. coli in a sample, which method comprises filtering the sample, if necessary after dilution, through a membrane filter having a pore size small enough to trap cells of any such strains present in the sample and made of a material not appreciably inhibitive of the passage therethrough of extracellular protein, and incubating the resulting used membrane filter with a selective medium which contains in an amount of at least about 0.1% by weight a surface active agent selective for Gram-positive microorganisms and which also contains lactose in a concentration no greater than 5 g/litre and is permissive of heat labile toxin-production by E. coli, if the thus-incubated membrane carries lactose-fermenting colonies placing the said membrane on a medium containing antitoxin to E. coli heat labile toxin and in which a complex of the said toxin and antitoxin is insoluble and incubating the said membrane and antitoxin-containing medium to permit toxin migration into the antitoxin-containing medium and the production of zones of precipitation corresponding to any colonies of heat labile toxigenic E. coli organisms on the said membrane.

2. A method as claimed in claim 1, wherein the sample is a food sample which is diluted before filtering.

3. A method as claimed in claim 1, wherein the sample is a water sample.

4. A method as claimed in claim 1, wherein the sample is a faeces sample which is diluted before filtering.

5. A method as claimed in any one of claims 1 to 4, wherein the membrane filter has a pore size of 0.1 μm to 0.65 μm, highly preferably 0.45 μm.

6. A method as claimed in any one of claims 1 to 5, wherein the membrane filter is a cellulose acetate membrane.

7. A method as claimed in any one of claims 1 to 6, wherein the selective agent is Tergitol, sodium lauryl sulphate, bile salts or Teepol.

8. A method as claimed in claim 7, wherein the selective agent is Tergitol in an amount of 0.1% by volume, sodium lauryl sulphate in an amount of 0.1% by weight, bile salts in an amount of 0.1% by weight, or Teepol in an amount of 0.4% by volume.

9. A method as claimed in claim 7, or claim 8, wherein the selective medium also contains an indicator.

10. A method as claimed in claim 9, wherein the indicator is Phenol Red or Cresol Red.

11. A method as claimed in any one of claims 1 to 10, wherein the selective medium contains 30 g ± 1 g of Tryptone soy Broth or Trypticase soy broth per 1000 ml and at least 4% by weight lactose.

12. A method as claimed in claim 11, wherein the selective medium also contains per 1000 ml approximately 12 g of yeast extract and/or approximately 0.2 g of Cresol Red indicator.

13. A method as claimed in any one of claims 1 to 12, wherein the antitoxin-containing medium contains an antiserum derived from an inoculation procedure using cholera toxin.

14. A method as claimed in claim 13, wherein the antitoxin-containing medium is an antitoxin agar containing the antiserum, purified agar and Tryptone soy broth or Trypticase soy broth.

15. A method as claimed in claim 14, wherein the Tryptone soy broth or Trypticase soy broth is present, per 1000 ml of medium, in an amount of 30 g ± 1 g, the purified agar is present in an amount of 1.5% ± 0.2% by weight and the antiserum is present in an amount of 0.5% ± 0.1% by volume.

16. A method as claimed in claim 15, wherein the antitoxin-containing medium also contains phosphate buffer and approximately 12 g of yeast extract per 1000 mol of medium.

17. A method as claimed in any one of claims 1 to 16, wherein antitoxin precipitation is enhanced by incorporating in the antitoxin-containing medium polymixin B or lincomycin.

18. A method as claimed in any one of claims 1 to 17, wherein the incubations are effected at 44°C.

19. A method as claimed in claim 1 and substantially as hereinbefore described.

20. A medium selective for E. coli and adapted for use in a method as claimed in claim 1 and comprising Tryptone soy broth or Trypticase soy broth in an amount of 30 g ± 1 g per 1000 ml of medium, Tergitol in an amount of 0.1% by volume or sodium lauryl sulphate in an amount of 0.1% by weight or Bile sales in an amount of 0.1% by weight or Teepol in an amount of 0.4% by volume, lactose in an amount of from 4 to 5 g per litre and Cresol Red in an amount of 0.2 g per litre.

21. A medium as claimed in claim 20 and also containing approximately 12 g of yeast extract per litre.

22. A medium as claimed in claim 20 or claim 21 and having an adjusted pH of 8.5.

23. An antitoxin-containing medium adapted for use in a method as claimed in claim 1 and comprising Tryptone soy broth or Trypticase soy broth in an amount of 30 g ± 1 g per 1000 ml of medium, purified agar in an amount of 1.5% ± 0.2% by weight and antiserum containing antitoxin to E. coli heat labile toxin in an amount of 0.5% ± 0.1% by volume.

24. A medium as claimed in claim 23 and also containing approximately 12 g of yeast extract per 1000 ml of medium.

25. A medium as claimed in claim 23 or claim 24 also containing phosphate buffer.

26. A medium as claimed in claim 20 and substantially as hereinbefore described.

27. A medium as claimed in claim 23 and substantially as hereinbefore described.

28. A kit adapted for use in the detection of heat labile toxigenic strains of E. coli comprising a membrane filter having a pore size of 0.1 to 0.65 μm, a medium as claimed in any one of claims 20 to 22 or 26 and/or a medium as claimed in any one of claims 23 to 25 or 27.

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